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1 **Glucose-mediated repression of plant biomass utilization in**
2 **the white-rot fungus *Dichomitus squalens***

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15 Running title: Glucose-mediated repression in *Dichomitus squalens*

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22 Keywords: *Dichomitus*, carbon catabolite repression, CAZymes, regulation

23

24 **Abstract**

25 The extent of carbon catabolite repression (CCR) at a global level is unknown
26 in wood-rotting fungi, which are critical to the carbon cycle and are a source of
27 biotechnological enzymes. CCR occurs in the presence of sufficient concentrations of
28 easily metabolizable carbon sources (*e.g.* glucose), down-regulating the expression of
29 genes encoding enzymes involved in the breakdown of complex carbon sources. We
30 investigated this phenomenon in the white-rot fungus *Dichomitus squalens* using
31 transcriptomics and exo-proteomics. In *D. squalens* cultures, approximately 7% of
32 genes were repressed in the presence of glucose compared to Avicel or xylan alone.
33 The glucose-repressed genes included the essential components for utilization of plant
34 biomass – Carbohydrate Active enZyme (CAZy) and carbon catabolic genes. The
35 majority of polysaccharide degrading CAZy genes were repressed and included
36 activities towards all major carbohydrate polymers present in plant cell walls, while
37 also repression of ligninolytic genes occurred. The transcriptome-level repression of
38 the CAZy genes observed on the Avicel cultures was strongly supported by exo-
39 proteomics. Protease encoding genes were generally not glucose-repressed indicating
40 their likely dominant role in scavenging for nitrogen rather than carbon. The extent of
41 CCR is surprising given that *D. squalens* rarely experiences high free sugar
42 concentrations in its woody environment and indicates that biotechnological use of *D.*
43 *squalens* for modification of plant biomass would benefit from de-repressed or
44 constitutively CAZymes-expressing strains.

Importance

White-rot fungi are critical to the carbon cycle because they can mineralise all wood components using enzymes that also have biotechnological potential. The occurrence of carbon catabolite repression (CCR) in white-rot fungi is poorly understood. Previously, CCR in wood-rotting fungi has only been demonstrated for a small number of genes. We demonstrated widespread glucose-mediated CCR of plant biomass utilisation in the white-rot fungus *D. squalens*. This indicates that the CCR mechanism has been largely retained even though wood-rotting fungi rarely experience commonly considered CCR conditions in their woody environment. The general lack of repression of genes encoding proteases along with the reduction in secreted CAZymes during CCR suggested that the retention of CCR may be connected with the need to conserve nitrogen use while growing on nitrogen-scarce wood. The widespread repression indicates that de-repressed strains could be beneficial for enzyme production.

Introduction

White-rot fungi break down all wood components, are critical to the carbon cycle and are a source of biotechnologically relevant enzymes (1). However, the extent of carbon catabolite repression (CCR) on plant biomass degradation in wood-rotting fungi is currently unknown. CCR occurs in the presence of sufficient concentrations of easily metabolizable carbon sources (*e.g.* glucose), down-regulating the expression of genes encoding enzymes involved in the breakdown of complex carbon sources. CCR has been studied mainly in ascomycete fungi (2) and has been examined at a

global level in various major groups of fungi, such as recently in anaerobic gut fungi (3), but not in wood-rotting fungi, such as *Dichomitus squalens*.

The analysis of glucose-mediated CCR in wood-rotting fungi is limited to small sets of transcripts and enzymatic activities. In the white-rot fungus *Phanerochaete chrysosporium*, glucose-mediated repression of two cellulase-encoding genes has been reported (4), while in the brown-rot fungus *Postia placenta*, glucose-mediated repression was observed for four hemicellulase, but not for four cellulase-encoding genes (5). In selected *Polyporales* white-rot fungi, cellulase and xylanase activities were significantly lowered when the cultures were supplemented with glucose (6).

Plant biomass utilisation by fungi requires the secretion of extracellular enzymes to degrade the polymeric components, transporters for the sugars released from the plant biomass and intracellular catabolic enzymes. There is evidence for catabolite repression of each of these functional categories in various ascomycete fungi (2) but not in wood-rotting basidiomycete fungi. Genes encoding other enzymes, such as proteases, that can scavenge for the scarce nitrogen in wood by hydrolyzing proteins (7), have been shown to be carbon catabolite repressed in ascomycete fungi (8-10), but there are reports indicating that protease activities in basidiomycete leaf-litter degrading fungi lack CCR (11). A transcriptional regulator of CCR, called CRE1/CreA, has been extensively studied in several ascomycete fungi (12), while other proteins, such as kinases, can mediate CCR independent of CRE1/CreA (13). Recently, *cre1* was deleted in the white-rot fungus *Pleurotus ostreatus* and exo-proteomics detected increased abundance of a subset of Carbohydrate Active enZymes (CAZymes) under apparently non-repressing

91 conditions (14). Analysis of the extent of glucose-mediated CCR can demonstrate if
92 there is a requirement to delete transcriptional or post-transcriptional regulators of
93 CCR in wood-rotting fungi for improved enzyme yields and/or biomass degradation.

94 In *D. squalens*, sugar inducers of polysaccharide degrading CAZyme encoding
95 genes have been identified (15) demonstrating an inducible, *i.e.* not constitutive,
96 system for these enzymes, which provides it with an ability to partially tailor its
97 molecular responses to wood composition (16). *D. squalens* has biotechnological
98 potential as a source of enzymes and for bioremediation and can be genetically
99 modified (17), making it a highly suitable species to analyse the effects of CCR. The
100 global effect of high glucose concentrations on the utilisation of plant biomass
101 components is not known in any wood rotting fungus. Occurrence of glucose-
102 mediated repression could be a tool to indicate whether *e.g.* particular CAZyme
103 families or expansin-like domain-containing proteins in wood-rotting fungi, are
104 involved in plant biomass degradation. In this study, we investigated the occurrence
105 and extent of glucose-mediated repression in the white-rot basidiomycete *D. squalens*
106 using two CAZyme-inducing polysaccharide substrates and time-points.

Results

Identification of carbon catabolite repressing conditions in *D. squalens*

Whether excess glucose could repress the secreted protein pattern from *D. squalens* cultures growing on ring-plates containing either Avicel or xylan was examined (Fig. 1A). The secreted proteins after 2 d, visualized using SDS-PAGE, showed a clear repression pattern on each substrate, albeit stronger on Avicel, and were analysed using exo-proteomics (Fig. 1B). Hierarchical clustering of the exo-proteins showed that the biological replicates from a condition generally clustered together (Fig. S1A). To investigate whether this repression extended beyond secreted proteins, the transcriptomes after 2 d as well as 5 h were analysed. Hierarchical clustering of all expressed genes showed two major time-dependent clusters (Fig. S1B). At the earlier time-point, the genes clustered by polysaccharide substrate, and presence and absence of glucose. In contrast at 2 d, transcripts from the Avicel-only cultures clustered separately compared to the other cultures from 2 d. Time, and not the type of polysaccharide or the presence of glucose, appeared to be the most dominant factor in the clustering, suggesting that gene induction and/or repression changed substantially over time.

Extensive glucose-mediated repression was observed at a global level

Glucose-mediated repression of *D. squalens* genes was extensive with approximately 7% of genes (1,042/15,295) repressed in one or more conditions (Table S1). A gene was considered glucose-repressed if the expression was > two-fold lower in the presence of glucose, the $P_{\text{adj}} < 0.05$ and the FPKM > 10 in the absence of glucose. A

129 large majority of the glucose-repressed genes were only repressed in one condition
130 (Fig. 2A). Not all genes were expressed in all these conditions, and a lack of induction
131 on one of the polysaccharides, preventing detection of repression, could explain why
132 approximately a quarter of the genes were found repressed on only one
133 polysaccharide (Fig. 2B). The remainder of the genes repressed on only one
134 polysaccharide were expressed on both, suggesting differences in how glucose-
135 mediated repression was functioning on each polysaccharide. On all four of the
136 repressed conditions, there was enrichment of GO terms representing processes
137 directly related to plant biomass utilisation, but also other processes (Fig. 2C and
138 Table S2). The GO term “hydrolase activity, hydrolyzing O-glycosyl compounds”
139 was enriched in glucose-repressed genes on both polysaccharides supplemented with
140 glucose compared to the polysaccharides without glucose. In addition, “sugar
141 transmembrane transporter activity” was enriched in the genes repressed on Avicel
142 supplemented with glucose compared to Avicel alone. Transport of the sugars
143 released from the polysaccharides is critical to growth on plant biomass and
144 enrichment in the glucose-repressed genes of GO terms for degradation as well as
145 transport demonstrates that repression of these two processes is coordinated. The GO
146 terms enriched among the genes repressed on Avicel supplemented with glucose at 2
147 d suggested repression of protein production from the GO terms “cellular amino acid
148 biosynthetic process” and “tRNA aminoacylation for protein translation”. Other GO
149 terms were enriched in repressed genes from a subset of the conditions and displayed
150 repression of biological functions beyond those directly related to plant biomass
151 degradation (Table S2). These included GO terms for lipase activity repressed on

xylan at 5 h, alcohol metabolic process on xylan at 2 d and organic acid biosynthetic process on Avicel at 2 d.

Glucose repressed a broad range of lignocellulose degrading activities

The *D. squalens* genome encodes 233 putative plant biomass degrading (PBD) CAZy genes and almost half (108) of these were glucose-repressed in at least one condition. The majority of the repressed PBD CAZy genes were repressed on only one of the two polysaccharides (Fig. 2B). This was due to either a lack of induction by released sugars on the other polysaccharide or differences in how the repression was being regulated, as more than half of these PBD CAZy genes repressed on only one polysaccharide were expressed substantially on both substrates without glucose. The glucose-repressed genes on Avicel were less affected by the time-point compared to xylan, where 2.5 times more CAZy genes were repressed on xylan at 2 d compared to 5 h. In addition to genes encoding CAZymes acting on polysaccharides, half (5/10) of the manganese peroxidase (MnP) encoding genes in *D. squalens* were repressed on xylan at 2 d. The total CAZy gene expression was five-fold higher on Avicel than on xylan at 2 d, and this likely contributed to approximately six-fold repression on Avicel compared to less than two-fold repression detected on xylan (Fig. S2). Hierarchical clustering also illustrated the higher CAZy gene expression on Avicel (Fig. 3 and Fig. S3). The most striking example of glucose-mediated repression was in Cluster 3, which contained mainly genes encoding cellulose-acting CAZymes that were highly expressed on Avicel at 2 d and strongly repressed with average 100-fold repression by glucose (Fig. 3). On xylan, the repressed genes mainly encoded xylan-acting enzymes that were side-chain rather than backbone acting, although in the exo-proteome, there

was one endo-xylanase protein lower produced in the presence of glucose at 2 d (Table S3). From the transcriptome analysis, it was clear that genes encoding a broad-range of activities were repressed including hemicellulolytic and pectinolytic activities.

Exo-proteomics identified 57 PBD CAZymes in at least one of the 2 d conditions (Table S3). Strikingly, the five most abundant proteins secreted into the Avicel ring-plates made up almost 80% of the total protein abundance, whereas in the ring-plates where Avicel was supplemented with glucose, these same proteins only made up approximately 10% of the total protein abundance. A protein was considered significantly lower produced in the presence of glucose if the abundance was > two-fold lower and the $P < 0.05$ or alternatively if only identified in the absence of glucose. There was a better positive correlation for Avicel than xylan when the proteins that were significantly lower produced in the presence of glucose were compared to the repressed genes. On Avicel at 2 d, almost all of the CAZymes that were lower produced in the presence of glucose also had the encoding gene repressed (18/19). In contrast, less than half of the CAZymes that were lower produced in the presence of glucose on xylan at 2 d had the encoding gene repressed (11/24).

Repression of catabolism of plant-derived sugars

Sugars are catabolised via several carbon catabolic pathways and intermediate metabolites can be inducers of genes encoding catabolic enzymes and CAZymes (18). Putative carbon catabolic enzymes in *D. squalens* were identified by homology to characterized enzymes from ascomycete fungi. Genes encoding 21 putative catabolic enzymes were glucose-repressed and half of these are involved in catabolism of

198 hemicellulose or pectin sugars, such as pentoses, mannose or galacturonic acid (Table
199 S1). Only a subset of the expressed genes from catabolic pathways related to
200 hemicellulose or pectin derived sugars (pentose catabolic pathway, Leloir pathway,
201 D-mannose pathway, D-galacturonic acid pathway and L-rhamnose pathway) were
202 glucose repressed at a time-point on Avicel or xylan. Repression of the entry point to
203 these pathways could repress the flux through the pathway obviating the need to
204 repress genes encoding subsequent enzymes, but the putative entry point to these
205 pathways was not preferentially repressed. Some carbon catabolic genes were higher
206 expressed in the presence of glucose, but these were not shared across the repressed
207 conditions. Glycolysis and the tricarboxylic acid (TCA) cycle catabolise glucose or its
208 derivatives in the main energy generating reactions of the cell. Surprisingly, few
209 glycolytic or TCA cycle genes were higher expressed in the presence of high glucose
210 concentrations and none at 5 h. In *Aspergillus oryzae*, many of the glycolytic and
211 TCA cycle genes are induced in glucose-rich compared to glucose-depleted
212 conditions (19). The general trend for glycolytic or TCA cycle genes was to be neither
213 induced nor repressed in the presence of excess glucose, but instead to remain
214 constitutively expressed. The same trend was observed for genes from the pentose
215 phosphate pathway (PPP), that catabolises glucose in parallel to glycolysis. This was
216 surprising because there could be a greater requirement for the ribose 5-phosphate
217 generated by the PPP for use in synthesising nucleotides in DNA replication if
218 replication was occurring more frequently at higher growth rates such as in the
219 presence of excess glucose. In contrast to the repression of *D. squalens* CAZy genes,
220 the magnitude of the repression was generally not greater than two-fold (Fig. S4) and

the proportion of the carbon catabolic enzymes encoding genes that were repressed (27%) was smaller than for CAZy genes (46%).

Limited glucose-mediated repression of proteases

Proteases function in scavenging for nitrogen during fungal wood degradation as the nitrogen content of wood is very low (7). The GO term for “serine-type peptidase activity” was enriched in the genes that were glucose-repressed on either of the polysaccharides at 2 d (Fig. 2C). However, only a small proportion of protease encoding genes (23/215) were repressed in at least one condition, mainly at the later time-point, while the expression of three-quarters of protease encoding genes was not significantly different (Table S1). This was reflected in the total abundance of transcripts encoding for proteases where the levels were not lower in the presence of excess glucose (Fig. 4). Exo-proteomics identified 26 proteases in at least one of the 2 d conditions (Table 1). There were only three proteases lower produced in the presence of glucose in the cultures growing on each polysaccharide whereas the majority of the proteases were not significantly lower produced in the presence of glucose. Indeed, the proteases were the other major functional category of secreted proteins, besides those degrading polysaccharides, secreted into the ring-plates in all conditions.

Candidate transcriptional regulator of glucose repression

Direct transcriptional repression via transcription factors binding to the promoters of the glucose-repressed genes likely plays a role in *D. squalens*. The putative *D. squalens cre1* ortholog, identified as the reciprocal BLASTP match of *P. ostreatus cre1* (14), was glucose-repressed on Avicel at 2 d, but was not induced by the high

glucose concentrations in the studied cultures. An alignment that included characterised CRE1/CreA proteins and putative CRE1 orthologs from three basidiomycetes, including *D. squalens*, showed conservation of the zinc binuclear cluster (Fig. S5). Surprisingly, there was little conservation between the ascomycete and basidiomycete proteins for the remainder of the sequence suggesting that how the transcription factor functions could differ substantially between the phyla. However, there was substantial conservation within each phyla for the proteins for the remainder of the sequence suggesting conservation of function within the phyla. The conservation of the zinc binuclear cluster supported analysis of the presence of the binding motif of CRE1/CreA that has been identified in ascomycetes (20, 21) in the promoters of the *D. squalens* glucose-repressed genes. There was a trend for enrichment (~ 30%) of motifs in glucose-repressed *D. squalens* genes, as measured by the mean number of motifs per gene promoter (Table 2 and Table S4).

Repression of candidate proteins with potential roles in biomass degradation

A gene being subject to glucose-mediated carbon catabolite repression when cultured with Avicel or xylan makes the encoded protein a stronger candidate for involvement in plant biomass degradation. The previous section showed how genes encoding proteins with well-established involvement in plant biomass degradation, such as cellulases, were strongly glucose-repressed thus allowing inferences to be made about other repressed genes.

β -glucuronidases from CAZy family GH79 cleave β -linked glucuronic acid residues in arabinogalactan-proteins (22), but these proteoglycans are a minor component of most lignocellulosic residues and thus a role for β -glucuronidases in

degradation of lignocellulose-rich residues is controversial. Recently, it was shown that β -glucuronidases can have a role in cellulose degradation, as polysaccharide monooxygenases catalyse oxidation of cellulose to glucuronic acid-containing oligosaccharides (23). Three *D. squalens* GH79 genes, two of which clustered closely with characterised β -glucuronidases from *Aspergillus niger* and *Neurospora crassa* (22) in phylogenetic analysis (Fig. S6), were repressed by glucose, further supporting a possible role for these proteins in lignocellulose degradation. The proteins from these three GH79s were detected with two of the three proteins lower produced in the presence of glucose (Table S3).

Previously, genes encoding expansin-like proteins were expressed when *D. squalens* was cultured with plant biomass substrates suggesting a role in the degradation (24). Expansin-like proteins such as the *Trichoderma reesei* SWO1 can disrupt the cellulose structure (25) and can boost the sugar release in saccharification of a non-pretreated grass substrate (26). In *D. squalens*, there was limited repression of genes encoding expansin-like proteins and their total expression was similar in absence or presence of glucose (Table S1). The only expansin-like protein detected in the exo-proteins was not lower produced in the presence of glucose (Table S3), but that does not exclude their role in plant biomass degradation, just indicates a different regulatory control of these genes.

Apart from plant biomass degradation, the gene encoding a *D. squalens* protein, FIP-dsq2 (Dicsqu464_1_PID_921554), which was reported to have immunomodulatory and anti-cancer properties (27), was glucose-repressed on Avicel at 5 h and 2 d as well as xylan at 2 d.

Discussion

We have demonstrated using transcriptomics and exo-proteomics widespread glucose-mediated repression in a wood-rotting basidiomycete fungus. Although high concentrations of free sugars are naturally rare for a wood-rotting fungus, they were insightful in suggesting that the existence of CCR in *D. squalens* was partly to conserve use of the scarce nitrogen in its woody biotope. There was widespread glucose-mediated repression of CAZy genes but not genes encoding nitrogen-scavenging proteases. Also, the pattern of repression provided support for direct and indirect mechanisms for how *D. squalens* reduced the expression of its CAZy genes.

The strong repression of secreted CAZy genes, but not genes encoding proteases, by glucose supplementation, suggests that CCR has been maintained by *D. squalens* to strongly conserve nitrogen use in its nitrogen-scarce woody biotope. It is possible that the sensing of increasing sugar concentrations signals successful breakdown of plant biomass and less of a need for high CAZyme secretion levels, which could be a drain on the limited nitrogen supply. Instead the scarce nitrogen could be diverted to support fungal growth and achieve a lower mycelial C:N balance than would be possible if a higher protein secretion level was maintained. It did appear that there was more growth in the presence of excess glucose as the mycelium was substantially thicker than when cultured on either polysaccharide in the absence of excess glucose. Previously, in three leaf-litter degrading basidiomycetes, the total protease activity was not lowered by glucose supplementation (11). The lack of repression in *D. squalens* is partly in contrast to the CCR of proteases that can be found in ascomycete fungi (10, 28, 29) possibly due to differences in the biotope or

lifestyle of the fungi. Regulation of proteases is a complex phenomenon and in particular the low nitrogen concentration in the culture medium, intended to replicate environmental conditions, may be a competing regulatory factor with any repressing effect of the glucose due to a physiological requirement of *D. squalens* to scavenge for nitrogen from proteins.

There were interesting temporal patterns to the induction and repression on both Avicel and xylan. On Avicel there was higher CAZyme encoding transcript levels at 2 d compared to 5 h and a three-time larger average fold-change of repression at the later time-point. The higher transcript levels at 2 d could be explained by the induction taking longer than 5 h to peak but the possibility cannot be excluded that the low concentrations of glucose released from Avicel at 5 h were sufficient to repress. On xylan at 2 d, the correlation between the CAZymes lower produced in the presence of glucose and repressed genes was ~ 50%, whereas on Avicel the correlation was ~ 95%. The lack of repression of the genes encoding for half of the CAZymes lower produced on xylan in the presence of glucose suggested that the repression of the genes occurred earlier than two days and had now ceased. Secondary (indirect) effects could also be contributing to the repression of these genes.

Whether the repression effect is primary (direct) or secondary (indirect) is difficult to ascertain. The presence of a *D. squalens* CRE1 ortholog and an enrichment of the CRE1/CreA binding motif in the promoters of the repressed genes suggest that there was a direct repression effect at the transcript level. In addition, at least two indirect repression effects were also suggested by the datasets. The enriched GO terms for “tRNA aminoacylation for protein translation” and “cellular amino acid

337 biosynthetic process” in the Avicel cultures supplemented with glucose suggest an
338 indirect effect leading to less exo-proteins due to less protein synthesis and translation
339 in the presence of glucose. The genes encoding β -glucosidases were glucose-
340 repressed mainly at 5 h on Avicel and the three β -glucosidase proteins detected were
341 not found to be lower produced in the presence of glucose. These non-repressed β -
342 glucosidases may indirectly contribute to an apparent repressing effect by reducing
343 the induction by means of hydrolysis of cellobiose, which was previously found to be
344 a major inducer of *D. squalens* CAZymes (15).

345 Analysis of glucose repression in orthologs of *D. squalens* glucose-repressed
346 genes of the white-rot fungus *P. chrysosporium* (4) and the brown-rot fungus *P.*
347 *placenta* (5) revealed similar and contrasting repressing trends. The *D. squalens*
348 orthologs of the cellobiohydrolase *Pc_cel6A* and cellobiose dehydrogenase *Pc_cdh*
349 were also glucose-repressed on Avicel at both time points, while the β -glucosidase
350 *Pc_bgl3A* was not repressed in *P. chrysosporium*, but the *D. squalens* ortholog was
351 repressed on Avicel at both time-points (see Table S1 for the IDs of the *D. squalens*
352 orthologs). The 30-fold lower concentration of glucose used by H. Suzuki et al. (4)
353 may be a factor in the lack of repression compared to *D. squalens*, as well as the lack
354 of an inducing carbon source in their study. In *P. placenta*, four cellulase genes
355 analysed by quantitative PCR were not found to be repressed by glucose compared to
356 a control without a carbon source (5), while in our study *D. squalens* orthologs for
357 three of these genes (β -glucosidase *Pp_bgl1*, and two endoglucanases *Pp_cel5B* and
358 *Pp_cel12A*) were repressed by glucose on Avicel at both time-points. Four *P.*
359 *placenta* hemicellulose encoding genes were repressed by glucose (5) and the *D.*
360 *squalens* orthologs of these were also repressed (β -xylosidase *Pp_bxl1*, β -mannanase

Pp_man5A and endo-xylanases *Pp_xyn10A-1* and *Pp_xyn10A-2*). Both of these studies (4, 5) consider glucose repression compared to a control without a carbon source whereas in our study, the control is an inducing polysaccharide.

It is somewhat surprising that high glucose concentrations had a widespread physiological effect in a wood-rotting fungus that would rarely experience such high concentrations in its woody environment, as recalcitrant lignocellulose degradation is a slow process occurring over several years (30). Glucose could potentially be sensed from catabolic intermediates that are also intermediates from catabolism of other sugars. *D. squalens* is predominantly found on softwoods (31) where galactoglucomannan, containing a backbone of glucose and mannose, is the main hemicellulose. In ascomycetes, mannose can be catabolised in two steps to D-fructose-6-phosphate that is the same intermediate that glucose is catabolised to (18). Thus D-fructose-6-phosphate accumulation could be the signal for excess mannose release more readily than other plant biomass derived sugars such as xylose which are catabolised by more complex pathways. There is evidence for cross-talk between cellulose and mannan sensing pathways in *N. crassa* (32), although the levels of other sugars besides glucose, such as mannose, are also likely to be released slowly from the wood.

Testing lower glucose concentrations and investigating the repressing effect of other sugars was beyond the scope of this study. Also, investigating the repressing effect of high nitrogen concentrations on protease expression and production would be of interest as our study used media with a low nitrogen concentration. The experimental conditions developed here facilitate screening based on protein secretion pattern or candidate CAZymes before more detailed transcriptome analysis. The

conditions also facilitate analysis of deletions of candidate regulatory proteins, such as the *D. squalens cre1* ortholog. Moreover, other *D. squalens* strains could be tested, as differences in CAZy gene induction between *D. squalens* monokaryotic and dikaryotic strains was reported previously (15).

In conclusion, for applications, de-repressed strains would be of benefit for a higher yield of CAZymes from *D. squalens*, particularly for the strongly repressed genes encoding cellobiohydrolases. Wood-rotting fungi seem to maintain the CCR physiological response found in other major fungal lineages despite the rarity of high free sugar concentrations in their natural biotope. The scarcity of nitrogen in wood may be a contributing factor to the maintenance of CCR.

Materials and Methods

Culturing conditions and biomolecule harvesting and extraction

D. squalens monokaryon CBS464.89 was maintained on malt extract agar (MEA) at 4°C and at -80°C in 30% glycerol. For pre-cultures, 0.5 cm plugs from a culture of *D. squalens* growing on MEA, was inoculated onto a 7.6 cm diameter perforated (0.1 µM) polycarbonate membrane (Maine Manufacturing) on top of low-nitrogen asparagine-succinate (LN-AS) agar (1.5% w/v) adjusted to pH 4.5 (33) containing either 1% w/v Avicel cellulose (Fluka) or beech wood xylan (Sigma) and incubated at 28°C for 3.5 d, after which the colony diameter was ~4.5 cm. The polycarbonate membrane containing the colony was then transferred to 9 cm ring-plates (34) containing LN-AS liquid medium containing the same polysaccharide (1% w/v) as the pre-culture plate or that polysaccharide supplemented with 3% w/v D-glucose. The rings in ring-plate are numbered from one to five from the centre to the outermost ring (see (34) for image with numbering of the rings). The well of each ring has a depth and width of 0.5 cm and is separated from adjacent rings by a barrier of 0.1 cm. After 5 h or 2 d from the transfer, the mycelia growing above rings two to five were sampled for RNA extraction. Also, 2 d after transfer, the liquid in rings two to five were pooled (for SDS-PAGE and exo-proteomics analysis), flash frozen in liquid nitrogen and stored at -20°C. LN-AS medium or agar containing Avicel or beech wood xylan was autoclaved at 121°C for 15 min, and the filter-sterilised vitamin solution was added after autoclaving. A 20% w/v glucose stock solution was sterilised using a membrane filter.

Total RNA was extracted from mycelium, purified and checked for quality as described previously (35). The mycelium was ground and mixed with Trizol reagent (Invitrogen) after harvesting and stored at -80°C before proceeding with remaining steps of RNA extraction.

Transcriptomic sequencing and analysis

Plate-based RNA sample preparation was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using the Illumina TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina: https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html, and with the following conditions: total RNA starting material was 1 µg per sample and eight cycles of PCR was used for library amplification. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flow cell, and following a 2x150 indexed run recipe.

Using BBduk (<https://sourceforge.net/projects/bbmap>), raw reads were evaluated for artifact sequence by kmer matching (kmer = 25), allowing one mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases

or one third of the original read length – whichever was longer). Filtered reads from each library were aligned to the Dicsqu464_1 genome assembly (36) using HISAT2 version 2.1.0 (37). FeatureCounts (38) was used to generate the raw gene counts using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p --primary options).

Statistical analysis was performed using DESeq2 (39). Hierarchical clustering on the expressed PBD CAZy genes using the log2 FPKM values (+1) was performed in the R statistical environment using the gplots package with the Euclidian distance and complete linkage options selected. Transcripts were considered differentially expressed if the DESeq2 fold change was > 2 or < 0.5 and $P_{\text{adj}} < 0.05$ as well as the FPKM > 10 in at least one of the two conditions being compared. Transcripts with FPKM ≤ 10 were considered lowly (*i.e.* not substantially) expressed. The plant biomass degrading CAZy annotations (40) were the same as used previously (16). For protease annotations, the Merops (41) annotations from the Dicsqu464_1 JGI portal were used. The annotations for carbon catabolic enzymes in *D. squalens* were obtained by reciprocal BLASTP using characterized carbon catabolic enzymes from *Aspergillus* species. Gene ontology (GO) enrichment analysis was performed using BiNGO (42) using the default setting with “GO_full” selected for the ontology.

SDS-PAGE and protein LC-MS/MS sample preparation and analysis

The liquid from rings two to five from each ring-plate from the 2 d cultures was pooled before centrifuging at 3,200 x g for 1 h at 4°C to pellet any solid particles from the polysaccharide substrates. From Avicel-containing cultures, 4 mL was concentrated ~20-fold using Vivaspin 500 columns (5000 kDa molecular weight cut-

off, GE Life Sciences) by centrifuging at 15,000 x g, 4°C for 3 h. All of the liquid from the concentrated supernatants were then precipitated. For the xylan-containing cultures, 500 µL of the non-concentrated supernatants was precipitated.

Proteins were precipitated on ice for 1 h using twice the sample volume of a solution of 20% trichloroacetic acid, 20 mM DTT and 80% acetone, then centrifuged at 3,200 x g for 30 min at 4°C. The pellet was then mixed with a solution of 20 mM DTT and 80% acetone and incubated overnight at -20°C, then centrifuged at 3,200 x g for 30 min at 4°C. The air-dried pellet was re-suspended in 150 µL 0.25% w/v anionic acid labile surfactant (AALS I) (Protea Biosciences) solution (prepared in 200 mM ammonium bicarbonate pH 7.8).

From the protein samples re-suspended in the AALS solution, 7.5 µL was analysed by SDS-PAGE. A 4X loading buffer (0.1 M Tris-HCl, pH 6.8, 42% glycerol, 4% w/v SDS, 0.02% w/v bromophenol blue and 0.6 M β-mercaptoethanol) was used where samples were boiled for 2 min to denature the proteins, cooled on ice for 2 min and centrifuged at ~10,000 x g for 2 min to remove insoluble material. The proteins were separated using a 12% w/v acrylamide SDS-containing running gel along with a PageRuler Plus Prestained Protein Ladder with a 10 to 250 kDa size range (Thermo Fisher). The gels were silver-stained based on standard methods.

The objective of the proteomic analysis was to compare equivalent volumes of the culture supernatant. For the six samples from xylan-containing cultures, the same volume of each sample with digested with trypsin followed by analysing the same volume of cleaned-up digests by LC-MS/MS. For the samples from Avicel-containing cultures, a three times larger volume was digested from the Avicel cultures

supplemented with glucose followed by analysing the same volume of cleaned-up digests by LC-MS/MS. Protein samples were digested with trypsin for proteomic analysis as previously described (43). Dried peptide digest samples were solubilized in a solution of 5% acetonitrile, 0.1% formic acid and 4 fmol/ μ L of trypsin-digested bovine serum albumin (BSA) (Michrom Bioresources) used as internal standard. Five μ L from all twelve cleaned-up digest samples was analyzed by LC-MS/MS using an Easy-LC II Nano-HPLC system connected in-line with a Velos LTQ-Orbitrap mass spectrometer (Thermo Fisher). LC-MS/MS data peptide and protein identification was done using the *D. squalens* protein sequence databases obtained from the Joint Genome Institute Dicsqu464_1 generated gene models (36). Protein identification and quantification was performed using the Proteome Discoverer 2.2 (Thermo Fisher) precursor ion quantitation workflow. Normalized individual protein area values were expressed as a fold value of the protein area value determined for the BSA internal standard.

The values for the samples from Avicel cultures supplemented with glucose, where three times larger volume was used for the trypsin digests, were divided by three. This correction for dilution was suitable as the amounts of proteins analysed were within the linear range of detection as validated by comparing by LC-MS/MS three-fold dilutions of two of the samples. A minimum of two peptides matched to a protein with at least one peptide of unique sequence was considered sufficient for identification in the dataset. For a protein to be considered present in a condition, the requirements were an abundance measurement in at least two of the replicates for that condition. For an identified protein to be considered significantly higher or lower produced, the requirements were > 2-fold difference in mean abundance values and P

510 < 0.05 from a two-tailed heteroscedastic (assuming unequal variances) t-test of the
511 log2 transformed abundance values. Where it was not possible to calculate a P-value,
512 a protein was considered as only present in one of the two conditions being compared
513 if there was abundance measurements in ≥ 2 of the replicates for the condition where
514 the protein was present and an abundance measurement in ≤ 1 replicate for the other
515 condition. The remaining proteins in the dataset were categorised as not significantly
516 different or not present in that comparison.

517 **Identification and alignment of CRE1/CreA protein sequences**

518 The *P. ostreatus* CRE1 (14) sequence was used to identify putative CRE1/CreA
519 orthologs by reciprocal BLASTp at MycoCosm in *D. squalens* (Dicsqu464_1), *P.*
520 *chrysosporium* (Phchr1) and *P. placenta* (PosplRSB12_1). The protein sequences of
521 the putative CRE1/CreA orthologs and the characterized CRE1/CreA from *P.*
522 *ostreatus* (14), *Fusarium oxysporum* (44), *Trichoderma reesei* (45), *N. crassa* (46)
523 and *Aspergillus nidulans* (47) were aligned using Clustal Omega (48) with default
524 parameters and visualized with Jalview (49). The conserved domain database (50)
525 was used to identify the zinc binuclear cluster domains which were annotated on the
526 alignment (Fig. S5).

527 **Promoter analysis for CRE1/CreA binding motifs**

528 Up to 1,000 bp length of promoter sequence upstream of the coding region of the
529 genes were obtained from the Dicsqu464_1 genome annotation (36). The reported
530 CreA binding motif 5'-SYGGRG-3', and its sub-motifs 5'-[GC][CT]GGGG-3' and
531 5'-[GC][CT]GGAG-3' were searched to both strands of promoter sequences as a
532 previous study (45) using an in-house Perl script.

533 **Accession numbers**

534 The reads from each of the RNAseq samples were deposited with the Sequence Read
535 Archive at NCBI with individual sample accession numbers (SRP215076, 215080-81,
536 215085, 215089, 215091-92, 215095, 215099, 215112, 215118-19, 215122-23,
537 215127, 215138, 215142, 215150-55 and 215157). The mass spectrometry data have
538 been deposited to the ProteomeXchange Consortium
539 (<http://proteomecentral.proteomexchange.org>) via the PRIDE (51) partner repository
540 with the dataset identifier PXD014774 and 10.6019/PXD014774.

541

542 **Declarations**

543 **Competing interests**

544 None declared.

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Tables

Table 1. Summary of proteases identified in the exo-proteomics and their differential production at 2 d after transfer of *D. squalens* onto ring-plates containing either Avicel or xylan with or without supplementation with 3% w/v D-glucose (G).

Dicsqu464_1 protein ID	Protease functional group (from Merops annotations)	Status Avicel vs Avicel+G	Status xylan vs xylan+G
153959	Aspartyl protease	No sig. diff.	No sig. diff.
542799		Higher in Avicel	Higher in xylan+G
812714		Only in Avicel+G	Higher in xylan
813173		No sig. diff.	No sig. diff.
941015		Only in Avicel+G	No sig. diff.
976858		Not present	No sig. diff.
1001160	Metalloprotease	Higher in Avicel	No sig. diff.
367116		Higher in Avicel+G	Higher in xylan
830406		No sig. diff.	No sig. diff.
907349		Higher in Avicel+G	No sig. diff.
910760		No sig. diff.	No sig. diff.
636882	Serine protease	No sig. diff.	No sig. diff.
806666		No sig. diff.	No sig. diff.
917108		No sig. diff.	No sig. diff.
917629		No sig. diff.	Not present
921007		Higher in Avicel+G	No sig. diff.
926265	Other	No sig. diff.	No sig. diff.
928513		Only in Avicel+G	No sig. diff.
929001		Higher in Avicel+G	Higher in xylan+G
977823		Higher in Avicel	Higher in xylan
1004022		No sig. diff.	No sig. diff.
917216		No sig. diff.	No sig. diff.
944074		No sig. diff.	No sig. diff.
953225		No sig. diff.	No sig. diff.
969286		No sig. diff.	No sig. diff.

733 Table 2. Summary of enrichment of CRE1/CreA binding motifs in the promoters of
734 *D. squalens* genes which were repressed in the presence of glucose on either Avicel or
735 xylan.

Type of motif	Motif sequence	Mean motif number per gene (all genes)	% enrichment of motifs in repressed genes			
			Avicel 5 h (325) ^a	Avicel 2 d (476) ^a	xylan 5 h (94) ^a	xylan 2 d (466) ^a
single direct	[GC][CT]GG[AG]G	1.48	+12.2	+14.3	+0.4	+16.1
single reverse	C[CT]CC[AG][GC]	1.78	+32.4	+26.2	+18.7	+38.4
singles all	both of above motifs	3.21	+21.4	+20.2	+8.7	+28.3
pair 1	[GC][CT]GG[AG]G[AGCT](1,100)[GC][CT]GG[AG]G	0.26	+19	+18.8	-9.5	+19.5
pair 2	C[CT]CC[AG][GC][AGCT](1,100)C[CT]CC[AG][GC]	0.34	+43.9	+42	+8.8	+49.1
pair 3	[GC][CT]GG[AG]G[AGCT](1,100)C[CT]CC[AG][GC]	0.25	+48.1	+24.9	+59	+44.1
pair 4	C[CT]CC[AG][GC][AGCT](1,100)[GC][CT]GG[AG]G	0.21	+38.2	+29.8	-0.1	+47.2
pairs all	all of above paired motifs	0.8	+29.5	+26.6	+13.9	+37.2

736 ^aIn parenthesis are the total number of genes that were repressed in that condition.

Figure legends

Fig. 1. Carbon catabolite repression phenotypes of *D. squalens* during growth on Avicel or xylan in the absence and presence of glucose. (A) Representative images of the *D. squalens* cultures on ring-plates containing either Avicel cellulose or beech wood xylan with or without supplementation with 3% w/v D-glucose (G) from either 5 h or 2 d after transfer of the pre-culture and (B) SDS-PAGE showing the banding pattern two days after transfer of pre-culture from protein samples used for proteomic analysis.

Fig. 2. Widespread glucose-mediated repression affecting a broad range of biological functions in *D. squalens*. (A) Comparison of the number of transcripts repressed by glucose (G) on Avicel or xylan after 5 h or 2 d of transfer from pre-culture. (B) Number of genes that were glucose-repressed on only one polysaccharide at a certain time-point. Each bar is divided into the proportions of these genes that were substantially expressed on both or only on the polysaccharide where glucose repression was detected. (C) Selection of enriched gene ontology (GO) terms showing broad effects of glucose-mediated repression. The size of the coloured bar indicates the percentage (for 100%, the bar would fill the entire cell) of the total genes annotated with an enriched GO term that were present in the repressed genes. (D) Comparison of plant biomass degrading CAZy genes repressed by glucose on Avicel or xylan after 5 h or 2 d of transfer of pre-culture.

Fig. 3. Repression of CAZymes affects a broad range of activities with strong support for the repressing pattern in the exo-proteomics. Hierarchical clustering, using

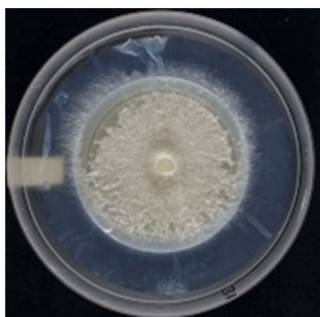
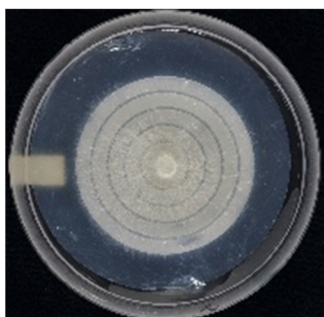
759 Euclidian distance, of transcript levels of plant biomass degrading CAZy encoding
760 genes from *D. squalens* mycelia grown on either Avicel or xylan with or without
761 supplementation with 3% w/v D-glucose (G). The CAZymes are colour-coded
762 according to substrate groups that they putatively act on (enzymes that act on multiple
763 polymers are coloured white). The protein abundance from exo-proteome is shown
764 adjacent to the transcript. See Fig. S3 for an image of the heatmap displaying
765 information about the gene each row corresponds to

766 Fig. 4. Total abundances of transcripts encoding proteases at 5 h and 2 d after transfer
767 of *D. squalens* onto ring-plates containing either Avicel or xylan with or without
768 supplementation with 3% w/v D-glucose (G). Error bars represent standard errors (n =
769 3).

A

Avicel

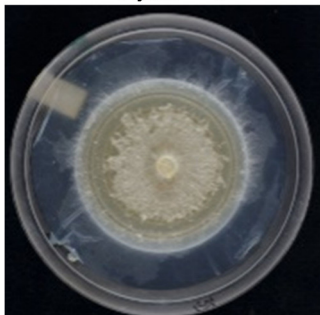
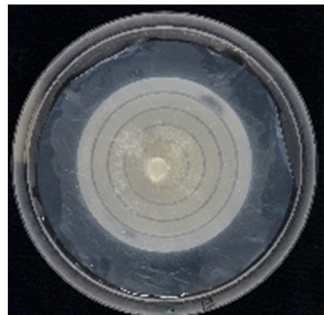
Xylan



5 h

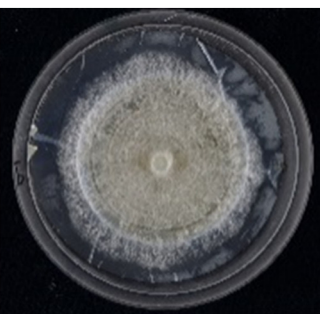
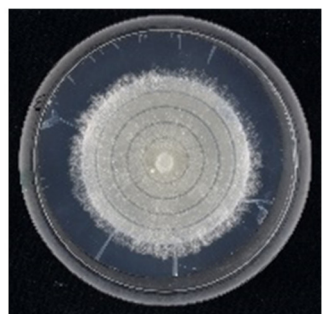
Avicel + G

Xylan + G



Avicel

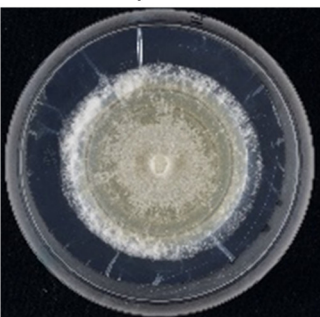
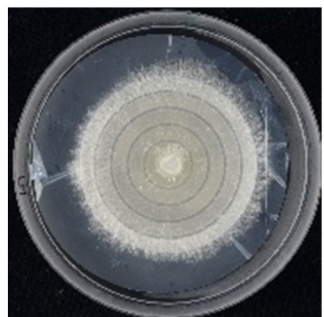
Xylan



2 d

Avicel + G

Xylan + G



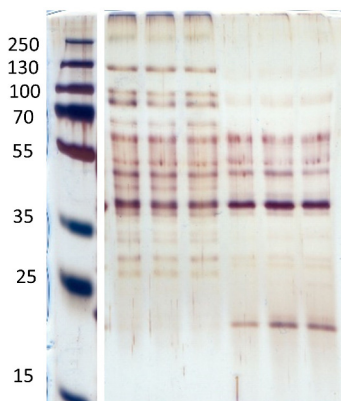
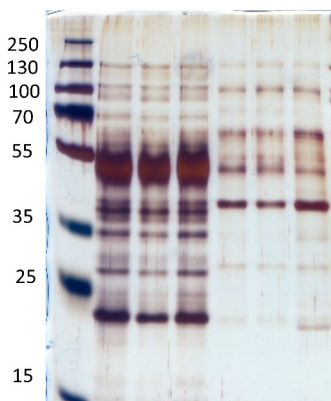
B

Avicel

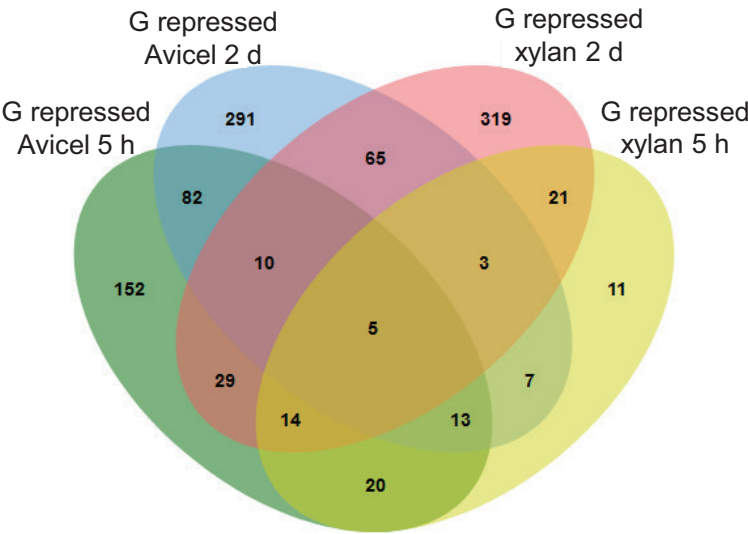
Avicel + G

Xylan

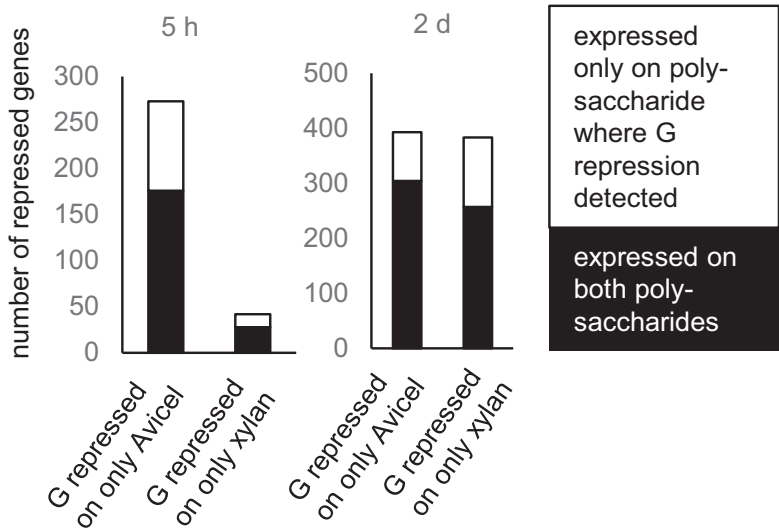
Xylan + G



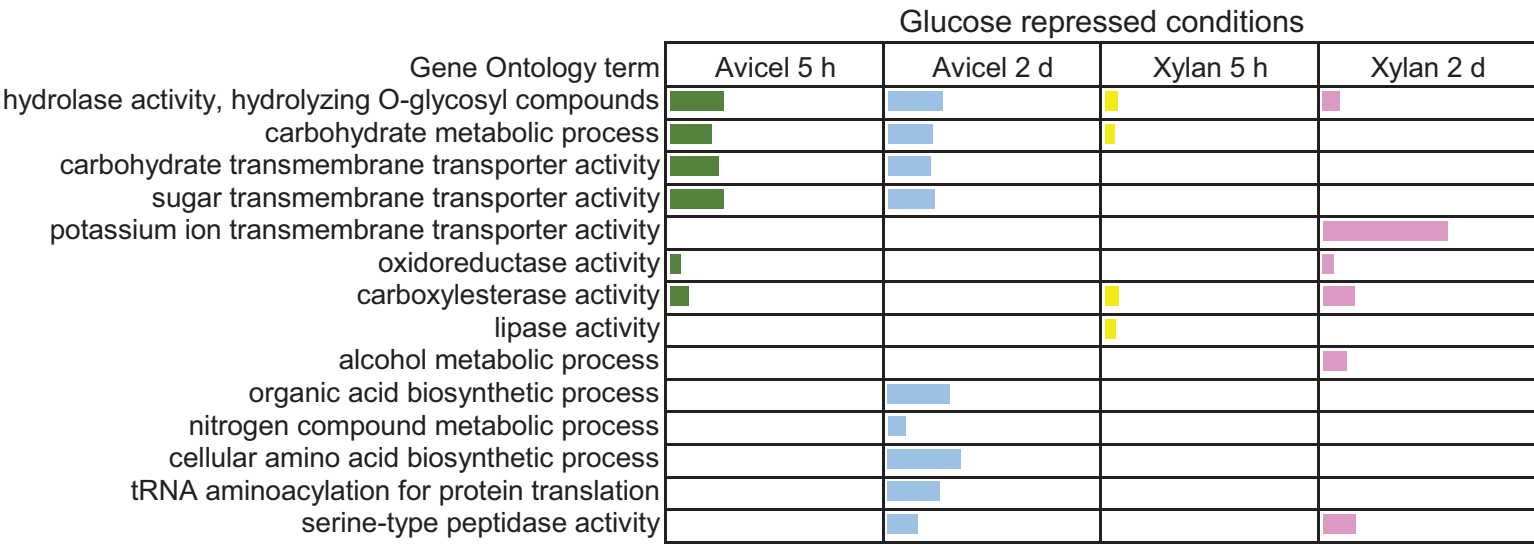
A



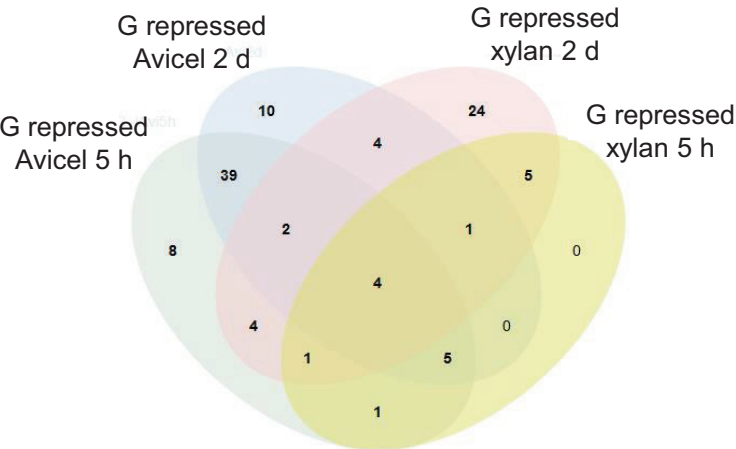
B



C



D



polymer colour-code

cellulose	multiple polymers
expansin-like	pectin
hemicellulose	starch
lignin	xylan
mannan	xyloglucan

\log_2 of abundance values

0 14

RNAseq

0 30

exo-proteome

Avicel 5h
Avicel + Glc 5h
Xylan 5h
Xylan + Glc 5h
Avicel 2d
Avicel + Glc 2d
Xylan 2d
Xylan + Glc 2d

Avicel 2d
Avicel + Glc 2d
Xylan 2d
Xylan + Glc 2d

polymer

